

## Variant proteins stimulate more IgM+ GC B-cells revealing a mechanism of cross-reactive recognition by antibody memory

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**Variant proteins stimulate more IgM+ GC B-cells revealing a mechanism of cross-reactive recognition by antibody memory**

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49    **Abstract**

50    Vaccines induce memory B-cells that provide high affinity secondary antibody  
51    responses to identical antigens. Memory B-cells can also re-instigate affinity  
52    maturation, but how this happens against antigenic variants is poorly understood  
53    despite its potential impact on driving broadly protective immunity against pathogens  
54    such as Influenza and Dengue. We immunised mice sequentially with identical or  
55    variant Dengue-virus envelope proteins and analysed antibody and germinal-centre  
56    (GC) responses. Variant protein boosts induced GC with higher proportions of IgM+  
57    B-cells. The most variant protein re-stimulated GCs with the highest proportion of  
58    IgM+ cells with the most diverse, least mutated V-genes and with a slower but  
59    efficient serum antibody response. Recombinant antibodies from GC B-cells showed  
60    a higher affinity for the variant antigen than antibodies from a primary response,  
61    confirming a memory origin. This reveals a new process of antibody memory, that  
62    IgM memory cells with fewer mutations participate in secondary responses to variant  
63    antigens, demonstrating how the hierarchical structure of B-cell memory is used and  
64    indicating the potential and limits of cross-reactive antibody based immunity.

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## 75 **Introduction**

76 Antibody-based immunity is underpinned by memory B-cells that have undergone  
77 antibody somatic hyper-mutation (SHM) and selection for improved antigen binding  
78 in germinal centres (GCs) (MacLennan et al., 1997). Re-challenge with the same  
79 antigen stimulates a rapid, higher affinity, secondary antibody response.

80 Protective immunity to highly mutable viruses, like Dengue and Influenza, can  
81 be induced by vaccination but the high level of variation often leads to immune  
82 escape (Nabel & Fauci, 2010), leading to a focus on generating vaccine responses  
83 against conserved antigenic regions (Wu et al., 2010; Corti et al., 2011; Wang et al.,  
84 2015).

85 Memory B-cells of IgM and IgG isotypes can also re-instigate GCs after  
86 secondary exposure (Dogan et al., 2009; Pape et al., 2011; McHeyzer-Williams et al.,  
87 2015), but how this happens against variant antigens is poorly understood despite its  
88 potential impact on driving the most broadly protective immunity.

89 Several studies suggest diversity in the memory B-cell population, showing  
90 that cells can express IgM or IgG (Dogan et al., 2009; Pape et al., 2011), be mutated  
91 or non-mutated (Kaji et al., 2012) and have low affinities (Smith et al., 1997), but still  
92 persist in GCs (Kuraoka et al., 2016).

93 It has long been speculated that this diversity may facilitate the recognition of  
94 antigenic variants (Herzenberg et al., 1980; Pape et al., 2011; Kaji et al., 2012) which  
95 could stimulate secondary GCs derived from less mutated, naïve-like, memory B-cells  
96 that still had an advantage over naïve B-cells due to their increased numbers, pre-  
97 selected V-genes and lower activation thresholds (Good et al., 2007; Good et al.,  
98 2009).

99           By sequentially immunizing mice with the same or different Dengue-virus  
100 envelope proteins, and analyzing serum antibodies and GC B-cells, we provide  
101 evidence that supports the hypothesis that less developed memory B-cells are used in  
102 secondary responses to variant antigens.

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## 124   **Results**

125   *E-protein variants elicit secondary serum antibody responses with different speed and*  
126   *cross-reactivity*

127   We chose Dengue-3 envelope protein (E3) for all priming immunisations. Boost  
128   immunisations were performed 38 days later with identical E3 protein or variant E2 or  
129   E4 proteins which have 68% and 63% overall sequence identity with E3, respectively.

130         The cross reactivity of E3-primed mouse serum IgG correlated with sequence  
131   identity (Fig. 1A), and overall cross-reactivity also correlated (Fig 1B).

132         Boosting with homotypic E3 antigen induced a rapid antibody memory  
133   response with anti-E3 titres rising rapidly to day 7, and not increasing further (Fig  
134   1D). E-protein boosted antisera was not reactive with an irrelevant His-tagged protein  
135   (PR8 HA)(Fig 1C).

136         Heterotypic boosting with E2 induced a rapid and significant increase in anti-  
137   E3 titre, as might be expected if cross-reactive memory antibodies against the priming  
138   E3 antigen were recalled (Figure 1D), that did not increase further by day 17. E4  
139   boosting induced a modest but not statistically significant increase in the anti-E3 titre,  
140   even by day 17, showing the E4 variant boost had not induced a significant anti-E3  
141   antibody memory response, or the induced antibodies had a low affinity for E3 (see  
142   discussion).

143         The anti-E2 titre induced by the E2 boost increased about 120-fold by day 7  
144   (Figure 1E), and did not increase further by day 17, further indicating that E2 boosting  
145   induced a rapid memory-like serum IgG response against E2 derived from cross-  
146   reactive E3 primed memory B-cells. Conversely the anti-E4 titre, induced by E4  
147   boosting, rose significantly but to a lower level, about 20-fold, by day 7 (Figure 1F)  
148   and showed a further rise by day 17. A boost alone did not induce a detectable

antibody titre however, ('BO', Figure 1D) suggesting a role for memory B-cells of some type and/or cross-reactive T-cell memory, facilitating the E4 boost response.

*Increased levels of IgM+ GC B-cells with fewer mutations after variant protein boosting*

E3 and E2 boosting induced early GC B-cell levels similarly by day 7, to 4.5-5.5% of total lymphocytes, which then reduced by two-thirds by day 17 (Figure 2B). E4 boosting induced GC B-cell levels about a third as high, which then reduced similarly by about 60% at day 17, remaining 4-fold higher than controls.

Analysis of the proportion of IgM+ GC B-cells showed a highly significant trend at day 7 after boosting, with the proportion of IgM+ GC B-cells correlating with increasingly variant challenge (Figure 2C). This trend continued to day 17. The proportion of IgM+ B-cells was also consistent between individuals in an experimental group (Figure 2D).

Overall levels of VH mutations increased in all groups from day 7 to day 17 (Figure 2E), consistent with secondary affinity maturation. Sequences are available in Supplementary File 1.

There were lower levels of SHM in IgM+ GC B-cells 7 days after the variant boosts, particularly with the most variant protein E4, compared to the homotypic E3 boost (Figure 2F). Boosting with variant proteins, therefore, induced early GCs with increased proportions of IgM B-cells that had fewer VH mutations.

Analysis of the VH clonality of GC B-cells after E-protein boosts showed that almost every VH sequence was from a distinct B-cell clone (Figure 2G). These data also showed that the two variant boosts elicited different repertoires of VH. 40% of the VH sequences sampled at day 7 from E2 boosted mice were either VH14-3 or the

174 closely related VH14-4 (black dots, Figure 2G), suggestive of a secondary response  
175 more focused on a particular epitope (see discussion) Some of these VH were also  
176 present in the homotypic E3 boost day 7 samples. but neither were detected at day 7  
177 after E4 boosting (Figure 2G).

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179 *Changes in serum affinity/avidity after variant antigen boosting*

180 E2-variant boosting induced an immediate and significant increase in avidity by day 7  
181 (Figure 3A) which did not detectably change until perhaps day 32, although data  
182 variability is high. A modest but significant increase in serum affinity, however, was  
183 detected by day 17, with a further increase detected by day 32 (Figure 3C). We  
184 interpret this to mean that a relatively small portion of serum IgG underwent affinity  
185 maturation by day 17 in response to the E2 boost and was not detectable by the Urea  
186 avidity assay due to high variability and the high pre-existing IgG titres (Figure 1E),  
187 or other limitations of the Urea assay (Alexander et al., 2015). Boosting with the E4  
188 variant elicited slower increases in relative affinity and avidity, only detectable by day  
189 32, but by then representing an equivalent, if not greater, increase compared to that  
190 induced by E2 (Figure 3B & 3D)..

191

192 *Similar memory T-cell stimulation by variant Dengue E-proteins*

193 Memory T-cells are necessary for memory B-cell responses against haptens and viral  
194 proteins (Aiba et al., 2010; Hebeis et al., 2004). We found no evidence that the  
195 memory T-cell response to re-stimulation by variant E-proteins was any different  
196 from re-stimulation by E3 (Figure 3E). These data imply that a deficiency in T-cell  
197 recognition of these antigens cannot explain the differences in response to E2 and E4  
198 challenge, and supports the idea that either T-cell receptors can recognize antigenic  
199 peptides from regions with around 50% sequence difference (see discussion) or, more



likely, B-cells present peptides from different, more conserved regions than those their antibodies bind to.

### *The primary antibody and GC response to E4*

For comparison with the E4 boost response, we performed primary immunisations with E4 and analysed serum antibodies and GC B-cells at day 7 and day 17. Serum levels of anti-E4 IgG rose to a moderate level by day 17 (mean EPT = 3.6, Figure 4A), being less than seen after E4 boosting (Figure 1F). GC B-cell levels rose to a mean of 0.8% lymphocytes at day seven after E4 priming, half as much as after the E4 boost, then fell similarly to the post boost samples by around 60% by day 17 (Figure 4B). As with the E-boost GCs, the proportion of IgM<sup>+</sup> GC B-cells fell over time (Figure 4C) and levels of VH mutation in all B-cells and IgM<sup>+</sup> B-cells increased (Figure 4D & 4E). The median level of VH mutation in IgM<sup>+</sup> GC B-cells at day 7 after E4 priming is less (=2) than after E4 boosting (=3) suggesting, not conclusively, that GC Bells at day 7 after E4 boosting are memory derived. Antibody titres were insufficient to do a relative affinity competition ELISA and no 7M Urea resistant IgG was detected 7 or 17 days after E4 priming (data not shown).

### *IgM Antibodies from E4 boost GC show evidence of prior selection*

If E4 boost induced B-cells are memory derived the antibodies should show evidence of pre-selection by the E3 prime. We made 48 recombinant antibodies (rAbs), 38 of which were IgM (supplementary file 2), 24 from E4 primed mice (day 7 and day 17) and 24 from E4 boosted mice (day 7). Figure 4F and Supplementary File 2, show the results from the initial screen of all rAbs against E4, indicating that the efficiency of detection of positive binding (deemed as O.D. > 0.1, useful for subsequent titration)

225 was quite low but consistent with the 30-50% binding frequency of GC rAbs  
226 previously observed (Kuraoka et al., 2016), except for E4 prime day 7, which has only  
227 2/13 rAbs binding strongly enough to be titrated. This might be expected of antibodies  
228 from a day 7 primary response GC, and indicated they were overall of lower affinity.  
229 Other rAbs from this group showed evidence of weak binding (supplementary file 2),  
230 indicating that the rAb cloning efficiency for this group was not reduced and only the  
231 two strongest binders were above the ELISA titration threshold. All but one of the  
232 positive binding rAbs were IgM. Figure 4G shows the ELISA titration and Figure 4H  
233 the derived endpoint titres, which we are using as a proxy of affinity. A more strongly  
234 binding IgM rAb from E4 boost day 7, B5, and the only positive binding IgG1 rAb,  
235 G6, are indicated on Figure 4H. The positive-binding rAbs from E4 prime day 17  
236 show a higher affinity than those from prime day 7, consistent with affinity  
237 maturation. Six of the seven positive-binding IgM rAbs from E4 boost day 7 show a  
238 higher affinity than the two strongest binding IgM rAbs from E4 prime day 7. This is  
239 consistent with pre-selection by the E3 prime immunization, and also considering the  
240 higher proportion of rAbs with an anti-E4 O.D. > 0.1, implies the GC B-cells  
241 expressing these antibodies are memory derived. rAb affinities were generally low,  
242 which might be expected of IgMs particularly in early GCs. We estimated the K<sub>d</sub> of  
243 rAbs B5 and G6 (an IgG1) as around 150nm and 1µm respectively (see Methods).  
244 Other rAbs would be in the super-micromolar range. Figure 4I shows the cross  
245 reactivity of rAbs with E3. Binding to E3 correlates with binding to E4, but because  
246 of the generally low rAb affinities we suggest that the antibodies cannot discriminate  
247 between similar epitopes. The higher affinity of E4 boost rAbs B5 and G6, and  
248 binding to E3, suggest they may have genuine specificity for E3, thus consistent with

249 their derivation from anti-E3 memory. That rAb B5 is an IgM with only one VH (and  
250 one Vkappa) mutation, provides further support for the proposal of this study.

251

## 252 **Discussion**

253 The most variant protein we boosted with, E4, stimulated GCs with the highest  
254 proportion of IgM<sup>+</sup> cells and with the lowest levels of VH gene mutation, greater VH-  
255 gene diversity, and a slower, more specific, serum IgG response that resulted in  
256 equivalent if not higher affinity, compared to the heterotypic E2 boost. This response  
257 was higher than the primary response to E4. IgM rAbs cloned from E4 boost day 7  
258 GC showed a higher affinity for E4 than those from E4 primed day 7 GC, implying  
259 they were memory derived. This demonstrates that IgM memory cells with fewer  
260 mutations, from 'lower' levels of the memory compartment, participate in secondary  
261 responses to variant antigens, and further challenges the hypothesis that highly  
262 mutated, class-switched cells elicited by homotypic antigen boosting are a 'mirror' of  
263 the antibody memory compartment (Weiss & Rajewsky, 1990). The slower nature of  
264 the E4 boost response also suggests a lower level of immediate differentiation of  
265 memory cells into AFCs than seen with for example the homotypic or E2 response,  
266 and is consistent with reduced numbers of high affinity class-switched memory cells  
267 recognizing E4.

268       The serum antibody response to the closer variant, E2, was more rapid, more  
269 cross-reactive and evidenced some earlier affinity maturation. These observations are  
270 consistent with a response derived more from the 'higher' layers of the E3 specific  
271 memory compartment. The IgM<sup>+</sup> cells induced by E2 boosting have more mutations  
272 than after E4 boosting, indicating they are memory derived. As there are higher  
273 proportions of these IgM<sup>+</sup> GC B-cells, with fewer mutations relative to the homotypic

274 E3 boost, this provides further support for the hypothesis that IgM<sup>+</sup> B-cells with  
 275 fewer mutations furnish memory responses to variant antigens

276 Naïve B-cells may contribute to the IgM<sup>+</sup> GC B-cells we observe after E4  
 277 boosting, although the higher affinities of the rAbs from this group suggest many are  
 278 memory derived. Also, the slightly higher median level of VH mutation and the  
 279 higher levels of IgM<sup>+</sup> GC B-cells after E4 boosting (2x) compared to priming,  
 280 suggest IgM<sup>+</sup>memory B-cells are involved in the boost response consistent with the  
 281 well established presence of IgM<sup>+</sup> memory cells with few or no mutations (Dogan et  
 282 al., 2009; Pape et al. 2011; Kaji et al., 2012) and the known lower activation threshold  
 283 of memory B-cells in response to antigen (Good et al., 2007 & 2009).

284 Whilst E3 specific memory cells may be expected to increase the anti-E3 titre  
 285 when stimulated by a cross-reactive E4 boost, the small but not significant effect we  
 286 observe (Figure 1D) is consistent with the lowest affinity, least mutated, E3-specific  
 287 memory cells being stimulated by an E4 boost. Antibodies from such cells may,  
 288 therefore not add much to the already high, affinity matured, anti-E3 titre induced by  
 289 E3 priming. The 14-fold higher anti-E4 titre at day 7 after boost (Figure 1F) versus  
 290 day 17 after prime (Figure 4A) also argues for a significant contribution from B-cell  
 291 memory.

292 The fusion-loop epitope in domain 2 of the dengue envelope protein is 100%  
 293 conserved between strains and in humans, antibodies against this are prevalent in  
 294 cross-reactive secondary responses (Lai et al, 2013, Chaudhury et al., 2017). The E2  
 295 boost response is consistent with this effect, especially considering the restricted  
 296 clonality seen in VH sequences, but the low anti-E3 titre induced by E4 is not. A  
 297 recent study (Chaudhury et al. 2017) showed that the mouse response to recombinant  
 298 E-protein is predominantly focused on domain 3 of the protein, and so cross reactivity

299 with the fusion loop epitope (domain 2) should be less dominant. While E2 and E4 are  
300 68% and 63% overall identical to E3, in domain 3, a focus of mouse antibodies, they  
301 are 62% and 51% identical, a bigger difference in differences, helping explain the  
302 responses we observe here.

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## 326 **Materials and Methods**

### 327 *Animals, immunisations and antigens*

328 Female 8-11-week old BALB/c mice were purchased from Charles River, U.K.

329 Primary immunisations were intra-peritoneal (IP) with 25µg recombinant Dengue

330 envelope protein (Biorbyt) precipitated in alum with  $2 \times 10^7$  heat-killed *B.pertussis*.

331 Secondary immunisations were IP with 25µg recombinant Dengue envelope protein

332 (Biorbyt) dissolved in phosphate-buffered saline (PBS). At designated time points

333 mice were anaesthetized and bled for collection of serum and then humanely

334 sacrificed for collection of spleen cells. Dengue envelope (E) proteins were C-

335 terminal His-tagged and expressed in *E-coli* prior to purification. Dengue proteins

336 were tested for endotoxin by LAL assay (Fisher Scientific) and contained it at a low

337 level: E2, 5.4EU/µg; E3, 2.5EU/µg; E4, 3.1EU/µg. Endotoxin in this range does not

338 give a detectable physiological response in mice (Copeland et al., 2005).

339

### 340 *ELISA for serum and rAbs*

341 ELISA plates (Nunc Maxisorp) were coated overnight at 4°C with 1µg/ml protein in

342 0.1M bicarbonate buffer pH 9.3. Plates were washed three times in PBS/0.05%

343 Tween-20 (Sigma) (PBST) and blocked for 30mins at room temperature with

344 PBST/2% bovine serum albumin (BSA, Sigma). Plates were then washed three times

345 and incubated with serum dilutions in PBST/1.0% BSA for two hours at room

346 temperature. After three washes plates were incubated with alkaline-phosphatase

347 conjugated goat anti-mouse IgG (Sigma) for one hour at room-temperature, washed

348 three times and developed with pNPP substrate (Sigma) for one hour. Absorbance

was measured at 405nm. For the initial rAb screen, rAbs were incubated at 100 $\mu$ gml<sup>-1</sup> in PBST/1.0% BSA for 2 hours at room temperature on plates coated with E4 and blocked as above, and subsequently treated as above except with use of anti-human IgG second layer (Sigma). Background binding to plates was determined using binding of non-specific polyclonal human IgG at 100 $\mu$ gml<sup>-1</sup>, because the rAbs were expressed as chimeric constructs with human constant regions, and this was subtracted from the rAb O.D. Positive binding rAbs were deemed to be those with O.D. > 0.1 that could be subject to an ELISA endpoint titration. For the ELISA titration and endpoint analysis, doubling dilutions of positive binding rAbs, and polyclonal IgG background subtraction control, were used starting at 100 $\mu$ gml<sup>-1</sup>. Endpoint titre was set at O.D. = 0.1 and calculated using interpolation on Graphpad Prism. The assay was repeated using E3 coated plates to determine the rAB cross reactivity. The affinity (Kd) of rAbs B5 and G6 (the two strongest binding rAbs) was estimated from the inflection point of the ELISA titration curve as indicating 50% maximal binding, and on the assumption that at these higher antibody concentrations binding of rAB to immobilized antigen will have a minor effect on concentration of unbound rAb. We estimated the B5 inflection point to be at approximately 25 $\mu$ gml<sup>-1</sup> (= approx. 150nM) and the G6 inflection point to be just above 100 $\mu$ gml<sup>-1</sup> (= approx. 1 $\mu$ M)

368

#### 369 *Competition ELISA*

ELISA plates were coated as above with target protein, then washed, blocked and washed as above except the blocking was done at 37°C for one hour. Mouse serum samples were diluted in PBST/1% BSA to twice the concentration of the maximum dilution that gave an absorbance at 405nm =1.0 in ELISA to the target protein. Serial

374 six-fold dilutions of competitor protein were made in PBST/1% BSA, such that the  
375 highest concentration of competitor was 2.4µg in 30µl. 30µl of diluted serum was  
376 mixed with 30µl of each competitor protein dilution and incubated in a polypropylene  
377 96-well plate at 37°C for 1 hour. Serum/competing antigen mixture (50µl) was then  
378 added to each well of the target antigen coated plate and incubated at 37°C for one  
379 hour. Plates were washed as above and then 50µl of alkaline-phosphatase conjugated  
380 anti-mouse IgG (Sigma) was added to each well followed by incubation at 37°C for  
381 one hour. Plates were washed as above and incubated with 75µl per well of p-  
382 nitrophenyl phosphate substrate (Sigma) for one hour at room temperature.  
383 Absorbance was measured at 405nm. All individual serum dilutions were also reacted  
384 in the absence of competitor, against BSA, following the same incubation protocol.  
385 These background values were subtracted from the competition ELISA values  
386 obtained above. The readings were then normalized so that the samples with the  
387 maximum competitor dilution gave a value of 1.0

388

#### 389 *Urea Avidity ELISA*

390 Adapted from Puschnik et al., 2013. Assay plates were coated with antigen and  
391 blocked as for the ELISA protocol. 1/200 dilutions of serum in PBST/1% BSA were  
392 incubated on plates for 2 hours at room temperature. Wells were washed once with  
393 PBST, incubated for 10 minutes at room temperature with PBST or PBST/7M Urea,  
394 washed a further two times with PBST and then treated as for standard ELISA. The  
395 avidity index was calculated by dividing readings from 7M Urea treatment by  
396 readings from PBST-only treatment, after subtraction of background absorbance.

397



398 *Flow cytometry*

399 Whole spleen cell-suspensions were red-cell depleted with Pharm-Lyse (BD  
400 Biosciences) and incubated with anti-CD16/32 monoclonal antibody (Fc-block, BD  
401 Biosciences) for 15minutes at 4<sup>o</sup>C. Cells were then stained with APC anti-B220,  
402 BV421 anti-CD38, PE anti-CD95/Fas (all BD) and FITC anti-IgM (eBioscience) for  
403 45 minutes at 4<sup>o</sup>C. After washing, cells were re-suspended in PBS 5% FCS (Gibco)  
404 and analysed or single-cell sorted on a FACS Aria II (BD).

405

406 *GC B-cell antibody sequencing, cloning, expression and purification*

407 Single GC B-cells were sorted into half a 96 well PCR plate (less 3 control wells)  
408 containing 10µl of chilled 10mM Tris pH 8.0, 1U/µl RNAsin (Promega) and placed on  
409 dry ice then at -80<sup>o</sup>C. One-Step RT-PCR (Qiagen) was performed according to  
410 manufacturers instructions, by adding 15µl RT-PCR master mix, using first-round  
411 primer sets described in Tiller et al., 2009, with heavy-chain and kappa-chain primers,  
412 for 50 cycles, annealing at 53.6<sup>o</sup>C. Heavy-chain second-round PCRs were performed  
413 using 2µl first-round product and the nested/semi-nested primer sets from Tiller et al.,  
414 2009, with Hot Star Taq polymerase (Qiagen) for 50 cycles annealing at 56<sup>o</sup>C.  
415 Second round PCR product (4µl) was analysed on a 1.2% agarose gel. Successful  
416 PCRs were then Sanger sequenced. For this study the sequencing primer was the pan  
417 VH primer 5'MsVHE (Tiller et al., 2009) which leaves part of the 5' of FR1  
418 unsequenced. For this reason the FR1 sequence was not included in the analysis. VH  
419 sequence identification and SHM analysis was done using the IMGT V-Quest online  
420 platform. VH sequences are in Supplementary File 1. Further cloning, construction  
421 and expression of antibodies as chimeric IgG1 rAbs was done according to Tiller et  
422 al., 2009. Briefly, second round PCRs of in-frame VH and VK sequences were

423 repeated with V-gene specific primers that included a restriction site for sub cloning  
424 (Tiller et al., 2009). These PCR products were purified (Qiagen), restriction digested,  
425 purified (Qiagen) and ligated (instant sticky-end ligase, NEB) into the appropriate  
426 expression vector containing either human IgG1 or Kappa constant regions, prior to  
427 transformation into *E. Coli* NEB5-alpha (NEB). Expression constructs in transformed  
428 colonies were verified by sequence analysis prior to preparation of plasmid mini-  
429 preps (Qiagen). 293A cells were split and grown to 80% confluence in DMEM with  
430 ultra-low IgG FCS (PAN Biotech) in 150mm plates prior to replacement of medium  
431 with 20ml Panserin 293A serum free medium. 15ug each of matched VH and VKappa  
432 constructs were added to 2ml saline with 90ug PEI, briefly vortexed and rested for  
433 10mins. Transfection solution was added to plates and mixed gently. After 3 days  
434 medium was collected, centrifuged at 800g for 10mins to clear debris, and further  
435 medium added. After a further 3 days medium was collected, cleared of debris as  
436 before and pooled. 100ul protein-G sepharose (GE Healthcare) was added to  
437 supernatants and incubated with rocking overnight at 4 °C. Protein G sepharose was  
438 collected by centrifugation at 800g for 10 mins and transferred in PBS to a PBS  
439 equilibrated spin column (Bio-Rad). After 3 rounds of washing with 800ul of PBS,  
440 rAbs were eluted in two 200ul passes of 0.1M Glycine (pH2.9) into a tube with 40ul  
441 of 1M Tris pH 8.0, 0.5% Sodium Azide. Antibody concentrations were determined by  
442 O.D. on a Nanodrop instrument (Thermo) and corrected for an extinction co-efficient  
443 of 1.36.

444

#### 445 *T-cell proliferation assay*

446 Spleens were harvested from female BALB/c AnCrl mice 39 days after challenge.  
447 Splenocytes ( $5 \times 10^5$ ) were cultured in triplicate with the indicated concentration of E-

448 protein in X-VIVO 15 medium. Cells were cultured for 96 hours and 0.5  $\mu$ Ci of [ $^3$ H]  
449 thymidine was added to wells for 16 hours before measurement with a 1450  
450 MicroBeta counter (Wallac).

451

#### 452 *Statistics*

453 For statistical analysis sample sizes were chosen to address group size reductions that  
454 observe the ARRIVE guidelines. Cages of three mice were randomly allocated to  
455 treatment groups. These group treatments were independently biologically replicated  
456 to give a sample size of 6. Where statistical analysis was applied, data points were  
457 analysed with Levene's test for equality of variance and where violated they were  
458 subject to a two-tailed Students t-test for unequal variance, otherwise the two-tailed t-  
459 test for equal variance.

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572

## 573 **Figure Legends**

### 574 **Figure 1**

#### 575 **Serum antibody responses after boosting with Dengue envelope protein variants**

576 **A**, Cross-reactivity of E3 primed serum IgG with E-protein variants. Red bar shows  
 577 mean value. Serum used was from mice mock-boosted with PBS 37 days after E3  
 578 priming and obtained 7 days later; E3, Dengue-3 envelope protein; E2, Dengue-2  
 579 envelope protein; E4, Dengue-4 envelope protein; % identity, sequence identity  
 580 between E3 envelope protein and respective protein; end-point titre (EPT) values  
 581 plotted are log2 of 1/(end point dilution x 100), each unit increase represents a  
 582 doubling of titre. **B**, E3 primed mouse serum cross-reactivity with E2 versus E4. **C**,

583 Control. Anti-PR8 HA serum IgG titre of E3 day 7 boost serum. **D**, Anti-E3 serum  
584 IgG titre after boosting with respective proteins. Red bar shows mean value. n=6 from  
585 two independent experiments for each group except boost only, n=3; first set of data  
586 points reproduced from panel A for comparison; numbers 3,2 and 4 refer to serotype  
587 of Dengue-envelope protein used for boost; BO, adjuvant primed, E3 boosted,  
588 analysed 7 days later; Day, days after boosting. p-values calculated using two-tailed  
589 Students t-test after testing for equality of variance. **E**, Anti-E2 serum IgG titre after  
590 E2 boost. Red bar shows mean value. n=6 from two independent experiments for each  
591 group; labeling and statistics as for panel D. **F**, Anti-E4 serum IgG titre after E4  
592 boost. Red bar shows mean value. n=6 from two independent experiments for each  
593 group; labeling and statistics as for panel D.

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## 598 **Figure 2**

599 **GC B-cell levels, isotypes, VH mutation and clonality after boosting with E-**  
600 **protein variants**

601 **A**, FACS gating strategy used to identify and sort GC B-cells and determine isotype.

602 **B**, GC B-cell levels after E-variant boosting, expressed as % total lymphocytes; Red

603 bar shows mean value; numbers 3,2 and 4 refer to serotype of Dengue-envelope

604 protein used for boost; BO, boost only, adjuvant primed, E3 boosted day 37, analysed

605 7 days later; Day, days after boosting. **C**, % IgM+ GC B-cells, of total GC B-cells,

606 after boosting. Red bar shows mean value. n=6 from two independent experiments for

607 each group; labels as for panel B except % identity which refers to sequence identity

608 between E3 and other variants; p-values calculated using two-tailed Students t-test  
609 after testing for equality of variance. **D**, Levels of IgM+ and IgM- GC B-cells in  
610 individual boosted mice. **E**, Number of mutations detected in VH of all isotypes of  
611 GC B-cells, from n=3 mice except E4 boost day 17, n=2. Red bar is median value.  
612 VH region sequenced is CDR1 to FR3; labeling as panel B. **F**, Number of mutations  
613 detected in VH of IgM+ GC B-cells, from n=3 mice except E4 boost day 17, n=2. Red  
614 bar is median value. **G**, Clonality of sequences from single GC B-cells 7 days after  
615 boosting; colours indicate different mice in each group; thin sectors, unique  
616 sequences; thicker sectors two or three clonal sequences according to sector size;  
617 black dots, VH 14-3 or VH14-4 sequences; numbers in circles, number of sequences  
618 from that mouse; Identical VH clones had the same: V-gene, CDR3 length, J-gene, D-  
619 gene if assigned, D-reading frame, three or fewer differences in CDR3 amino acid  
620 sequence.

621

622

### 623 **Figure 3**

624 **Relative serum affinity and avidity after boosting with E-protein variants, and**

625 **T-cell re-stimulation**

626 **A**, Relative avidity of E2 boost serum for E2, measured by resistance to 7M Urea.

627 Red bar shows mean value; Day, days after E2 boosting; Day 0 sample was from mice

628 mock-boosted with PBS 37 days after priming with E3 and obtained 7 days later. **B**,

629 Relative avidity of E4 boost serum for E4, measured by resistance to 7M Urea.

630 Labeling as for panel A; Day 0 sample was from mice mock-boosted with PBS 37

631 days after priming with E3 and obtained 7 days later **C**, Relative affinity of E2

632 boosted serum for E2. Inhibition by lower concentration of competitor implies higher



633 affinity of serum for competitor. Maximum competitor amount 2 $\mu$ g in 50 $\mu$ l followed  
634 by six-fold dilutions of competitor; timepoint of samples and numbers of individuals  
635 in group indicated. Open circles, E2 boost day 17 serum competed with irrelevant  
636 His-tagged protein measured on E2 target **D**, Relative affinity of E4 boosted serum for  
637 E4. Labeling as for panel A. **E**, T-cell proliferation measured by  $^3$ H incorporation 96  
638 hours after re-stimulation in vitro with indicated amounts of E-protein variants; error  
639 bars indicate standard error of the mean; n=4 or 5 from two independent experiments  
640 (see source data). Closed symbols, E3 primed mouse splenocytes re-stimulated with  
641 indicated E-protein variant. Open symbols, adjuvant primed mouse splenocytes re-  
642 stimulated with indicated E-protein variant.

643

#### 644 **Figure 3 Source Data File**

645 Source data for Figure 3 panels C, D and E

646

647

#### 648 **Figure 4**

##### 649 **Primary response to E4 and rAb binding.**

650 **A**, anti-E4 IgG titre after E4 priming; Red bars show mean titres; **A**, serum from  
651 adjuvant-only primed mice at day 45; d7, 7 days after E4 priming; d17, 17 days after  
652 E4 priming; EPT, end-point titre calculated as for Figure 1. **B**, GC B-cell levels after  
653 E4 priming; Red bars indicate mean levels; **A**, cells from adjuvant-only primed mice 7  
654 days after priming; other x-axis labels as for panel A. **C**, %IgM+ GC B-cells after E4  
655 priming; Red bars show mean values; x-axis labels as for panel A. **D**, Numbers of VH  
656 mutations in all isotypes of GC B-cells after E4 priming; Red bars show median  
657 values, from n=3 mice (d7) and n=2 mice (d17); x-axis labels as for panel A. **E**,

658 Numbers of VH mutations in IgM<sup>+</sup> GC B-cells after E4 priming and boosting; Red  
659 bars show median values, from n=3 mice (d7), n=2 mice (d17) and n=3 mice E4Bd7;  
660 x-axis labels as for panel A except E4Bd7, 7 days after E4 boosting which was 38  
661 days after E3 priming. **F**, ELISA screen of binding of all 48 rAbs. rAbs incubated at  
662 100µgml<sup>-1</sup>. Number of rAbs in each group indicated. P7, 7days after E4 prime; P17,  
663 17 days after E4 prime; B7, 7 days after E4 boost. As the antibodies were cloned as  
664 chimeric human IgG1 antibodies the background from non-specific human polyclonal  
665 IgG binding has been subtracted from O.D. readings. Values in supplementary file 2.  
666 **G**, ELISA titration of rAbs that showed binding O.D. > 0.1 in panel F. All but one  
667 were IgM. IgG1 rAb indicated. Background subtraction as for panel F, using  
668 appropriate dilution of polyclonal IgG. **H**, Anti-E4 end point titre of positive-binding  
669 rAbs, used as a proxy of rAB affinity. X-axis labels as for panel F. End-point titre  
670 values plotted are log<sub>2</sub> of 1/end point dilution (undiluted = 100µgml<sup>-1</sup>). Red bars show  
671 median values (excluding any IgG1 data). Stronger binding IgM rAb 'B5', and IgG1  
672 rAb 'G6' EPT readings indicated. **I**, anti-E3 versus anti-E4 endpoint titres. Star, E4  
673 prime day 7 rAbs; Square, E4 prime day 17 rAbs; circle, E4 boost day 7 rAbs. IgG1  
674 EPT reading indicated. End-point titre values plotted are log<sub>2</sub> of 1/end point dilution  
675 (undiluted = 100µgml<sup>-1</sup>).

676

#### 677 **Supplementary File 1**

#### 678 **GC B-cell VH Sequences**

679 VH sequences from single sorted GC B-cells. Sequences are grouped into treatment  
680 groups, and within this, arranged in blocks for sequences from individual mice. Raw  
681 sequences were analysed by IMGT V-Quest. Due to cloning and sequencing primers  
682 being at start of FR1 region, this region not included in mutation analysis. CDR1T,

683 total mutations in CDR1; CDR1S, silent mutations in CDR1; CDR1R, replacement  
684 mutations in CDR1; likewise for FR2, CDR2 and FR3 regions; Tot Mut, total  
685 mutations in CDR1 to FR3 regions.

686

687 **Supplementary File 2**

688 Data on recombinant antibodies

689

Figure 1

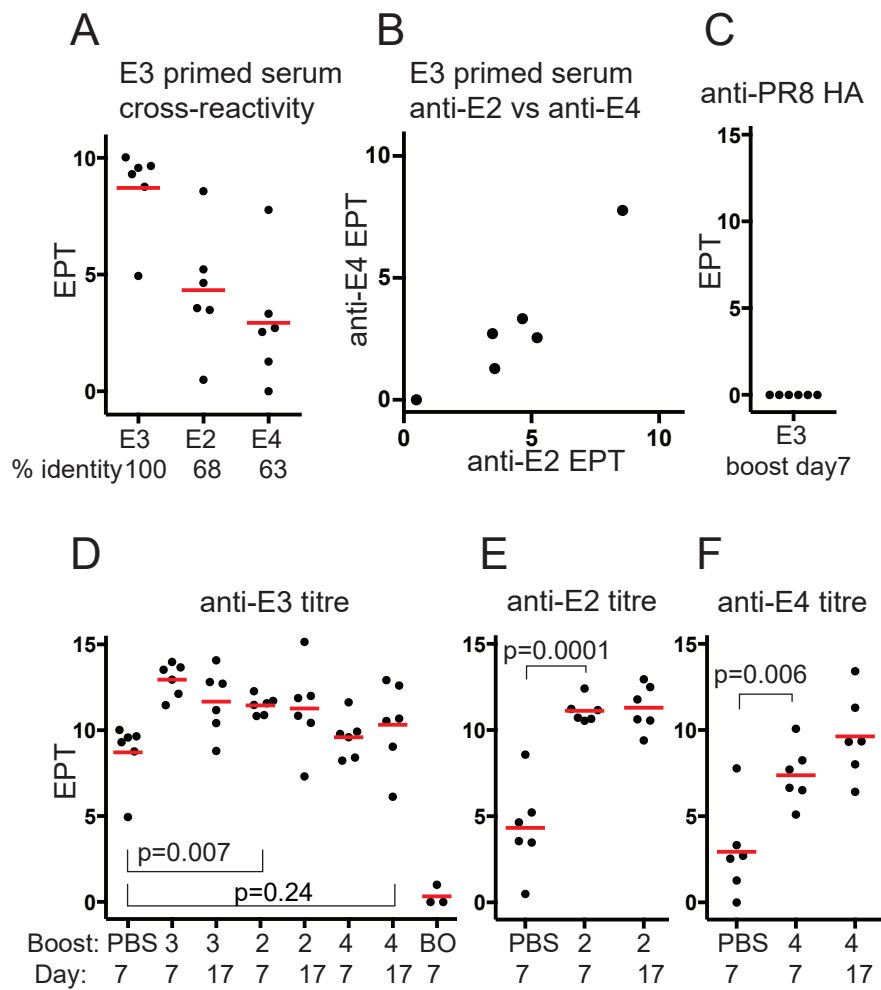
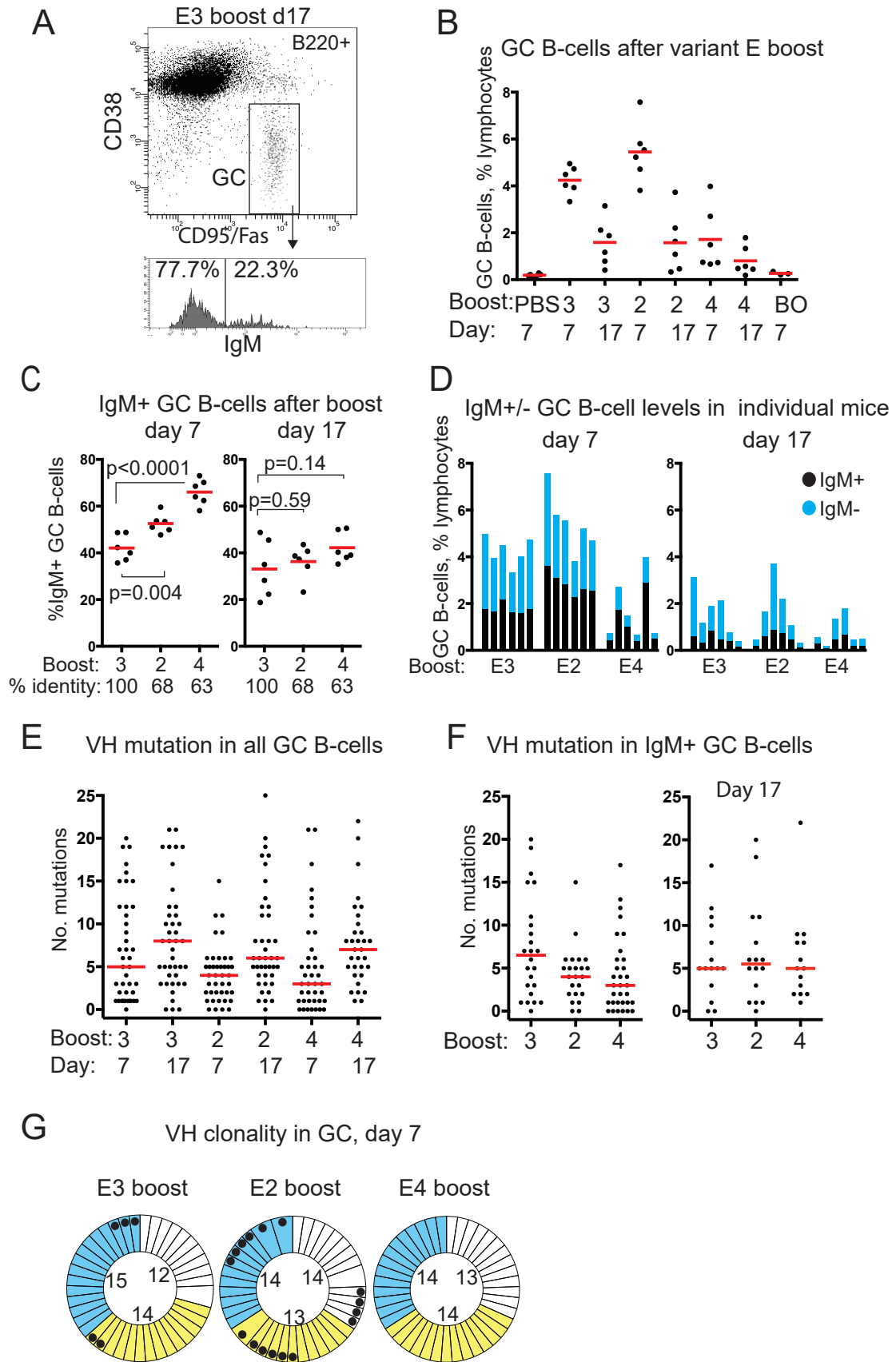


Figure 2



# Figure 3

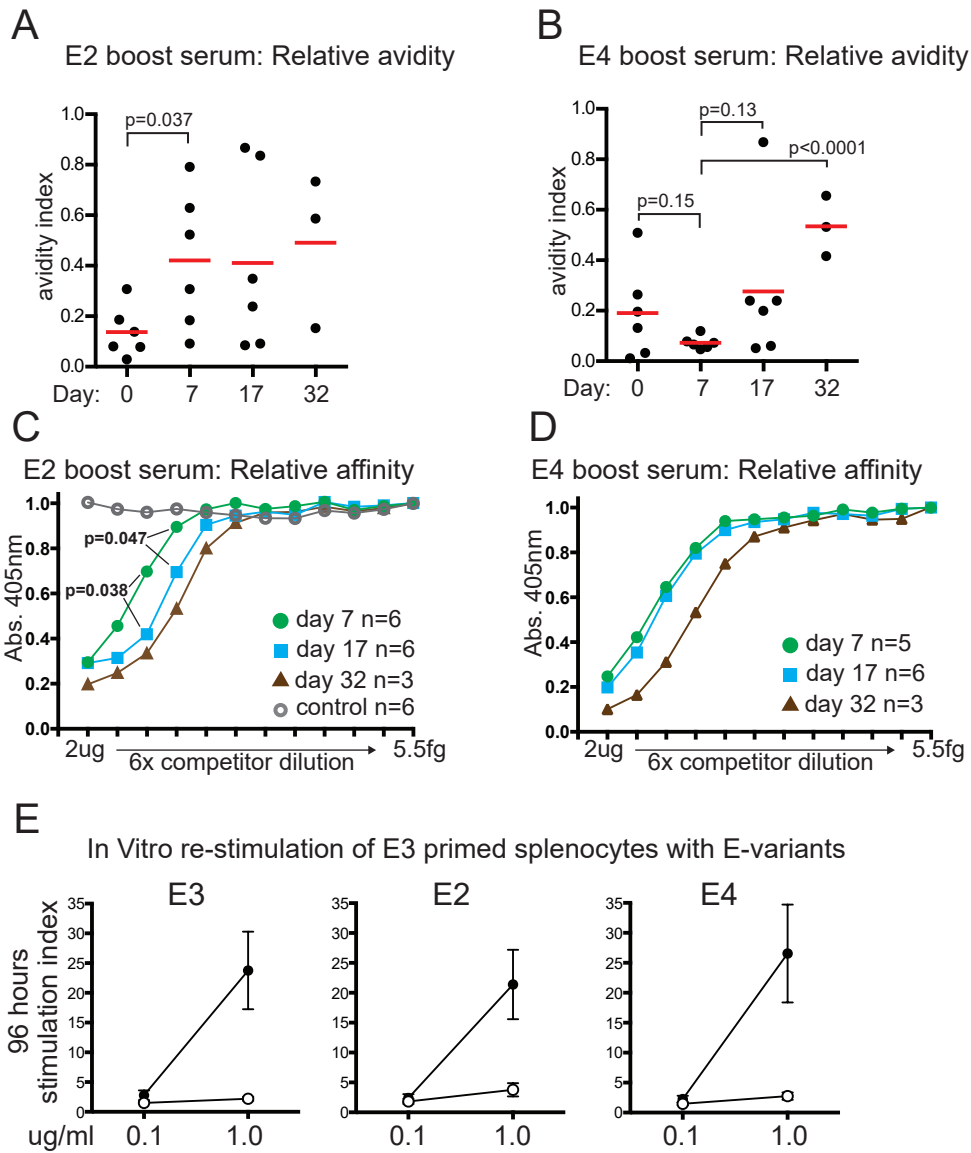


Figure 4

Primary Response to E4

